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TITLE: The Role of CRELD1 Isoform 9B in the Pathogenesis of Breast Cancer

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14. ABSTRACT  Purpose: The goal is to develop a mouse model that expresses isoform 9b in mammary tissues, and to determine if CRELD1-9b causes or predisposes the mice to develop breast tumors, or participates in cancer progression. Scope: This study will determine if expression of CRELD1-9b contributes to the cause and/or progression of breast cancer. Information from this study will be used to better understand the relationship of CRELD1-9b to breast cancer tumor biology, and to develop it as a new marker for early detection of breast cancer or breast cancer progression. Since utilization of exon 9b appears to be unique to cancer cells it is thought to participate in the malignant process and hence would be a potentially viable target for therapeutic intervention. Major findings: We have tested two different mammalian expression vectors for expression of the CRELD1-9b under the control of the mouse mammary tumor virus (MMTV) promoter. The promoter is inducible, giving us control over mammary tissue-specific expression. One of the vectors exhibited superior performance in expressing CRELD1-9b, with verified protein expression.					
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**INTRODUCTION:** *CRELD1* is a recently identified gene that encodes a protein thought to be involved in cell adhesion/migration [Rupp et al., 2002]. *CRELD1* resides on chromosome 3p25.3, coinciding with a locus for familial breast cancer. Investigation of alternative splicing of *CRELD1* led to the discovery of a cryptic exon embedded in intron 9 (exon 9b) that when utilized produces an alternatively spliced product CRELD1-9b. Transcripts utilizing exon 9b are expressed by breast cancer cells, but not by normal mammary tissue. Normally CRELD1 is tethered to the cell surface by a two pass transmembrane domain. Utilization of exon 9b produces an altered full-length molecule by eliminating the transmembrane domain and replacing it with a unique carboxyl-terminus. This results in a molecule that is completely secreted into the extracellular space as it is no longer tethered to the cell membrane. Since the extracellular domain of the molecule is unaltered we propose that CRELD1-9b will act as a “decoy” molecule, modulating the function of membrane-bound CRELD1 by competitively binding ligand and making it unavailable to the membrane bound isoform. Based on the expression of CRELD1-9b by breast cancer cells, and the coincident location of the CRELD1 gene with a breast cancer susceptibility locus, we hypothesize that expression of CRELD1-9b plays a role in the development or progression of breast cancer. The purpose of this study is to develop a mouse model that expresses isoform 9b in mammary tissues, and to determine if CRELD1-9b causes or predisposes the mice to develop breast tumors, or participates in cancer progression. The scope of this study is to develop tools to determine if expression of CRELD1-9b contributes to the cause and/or progression of breast cancer.

**BODY:** The following data was generated in the 2004-2006 grant period.

*Task 1.* Create a mammalian expression construct that will express *CRELD1* isoform 9b under the control of the mammary gland-specific whey acidic protein (WAP) promoter (Months 1-2).

- a. Clone isoform 9b cDNA by RT-PCR from cDNA isolated from breast cancer cells (QuickClone cDNA™, Clontech) into a shuttle vector, sequence to confirm integrity of the clone (Month 1).

Progress – Task 1a completed as follows:

Isoform 9b cDNA was isolated using RT-PCR and cloned into a PCR product TOPO™ shuttle vector (Invitrogen). Insert positive clones were identified by restriction digestion and bi-directionally sequenced to confirm the identity and integrity of the insert (Figure 1).

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gaattgcatcttatgcgccctttccccacccatccccacagcccctgcaatacccgagtttggcctcttttgccttgtaataacg
cagatcccagcgccacggcaccttagaacagacctttttctttctcgctggtgggctgactccttcagtgaaagcctctccac
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caccgccatcttcattggggctgtggcgccatgactggctactggttgtcagagcgcagtgaccgtgtgctggagggcttc  
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tggaactcaggacagcttgggtttatTTTTGAGAGTGGGGTAAAGCACCCTACCTGCCTTACAGAGCAGCCCAGGTACCCAG  
GCCCCGGCAGACAAGGCCCTGGGGTAAAAAGTAGCCCTGAAGGTGGATACCATGAGCTCTTCACCTGGCGGGGACTGGCAG  
GCTTCACAATGTGTGAATTTCAAAAGTTTTTCCTTAATGGTGGCTGCTAGAGCTTTGGCCCTGCTTAGGATTAGGTGGTCC  
TCACAGGGGTGGGGCCATCACAGCTCCCTCCTGCCAGCTGCATGCTGCCAGTTCCTGTTCTGTGTTCCACCACATCCCCACAC  
CCCATTGCCACTTATTTATTCATCTCAGGAAATAAAGAAAGGTCTTGAAAGTT

Figure 1. Sequence of isoform 9b cDNA coding region. The start (ATG) and termination (TGA) codons are underlined. The 5' and 3' untranslated regions are in lower case letters, the coding region is in upper case letters. The total cDNA length is 2.1 kb, encoding a 422 amino acid protein.

- b. Subclone isoform 9b cDNA into expression vector with WAP promoter (Month 2).

Progress – Task 1b completed as follows:

The cDNA was excised from the TOPO shuttle vector and subcloned into an expression vector under the control of the mammary gland-specific whey acid protein (WAP) promoter [Sternlicht et al., 1999] kindly provided by Dr. Werb. Clones with appropriate sized inserts were identified with restriction digestion.

- c. DNA sequence analysis to confirm the clone sequence and integrity (Month 2).

Progress – Task 1c completed as follows:

Five positive clones were sequenced to confirm the insert identity and integrity. All five clones had proper inserts.

- d. Appended sub-task d. Upon advice from the investigators that provided the vector, the CRELD1-9b constructs were transfected into cultured mouse mammary epithelial cells (ATCC) to test for expression potential. Northern blot analysis indicated that none of the clones were expressing CRELD1-9b (data not shown). Consequently, an additional 10 clones were selected and sequenced to verify the inserts. These clones were individually transfected into the mouse mammary cells. No expression of CRELD1-9b was detected from any of the clones. Transfection efficiency of the cells was tested by transfection with a *lacZ* expressing construct. Post-transfection  $\beta$ -galactosidase staining indicated that transfection efficiency was high (>80%), indicating that poor transfection was not a problem and that this vector system was not suitable for this application.
- e. Appended sub-task e. In order to complete Task 1 we investigated the availability of other suitable expression vectors. Vectors that utilize the mouse mammary tumor virus (MMTV) promoter have been successfully used to express exogenous gene products specifically in mouse mammary tissues. We identified sources for three different vectors that use the MMTV promoter and are suitable for transgene expression. The MkbpA II construct was provided by Dr. Jeffrey Rosen, Baylor College of Medicine [Li et al., 2003]. This vector construct contains the MMTV-LTR fragment, which is transcriptionally active when induced with dexamethasone. The pBS-SK-MMTV and pUC18-MMTV-TVA vectors were obtained from Dr. Dezhong Liao at Wayne State University. Both express exogenous inserts from the MMTV promoter under the control of dexamethasone. The CRELD1-9b insert was individually subcloned into each of these vectors downstream of the MMTV promoters. Due to the different cloning challenges presented by each vector, the MkbpA II vector construct (designated Mkbp-CRELD1-9b) was the first to be completed. Clones were selected as before and the inserts sequenced to confirm identity and integrity (data not shown).

- f. Appended sub-task f. DNA from selected Mkbp-CRELD1-9b plasmids was purified in sufficient quantities to perform expression assays using cultured mouse mammary epithelial cells as before. The cells were transiently transfected with the expression constructs and the cells culture in the presence (+) and absence (–) of dexamethasone to induce the promoter. Northern blot analysis was used to determine if induced *CRELD1-9b* expression could be detected. Figure 2 shows a northern blot displaying induction of *CRELD1-9b* expression from this construct.

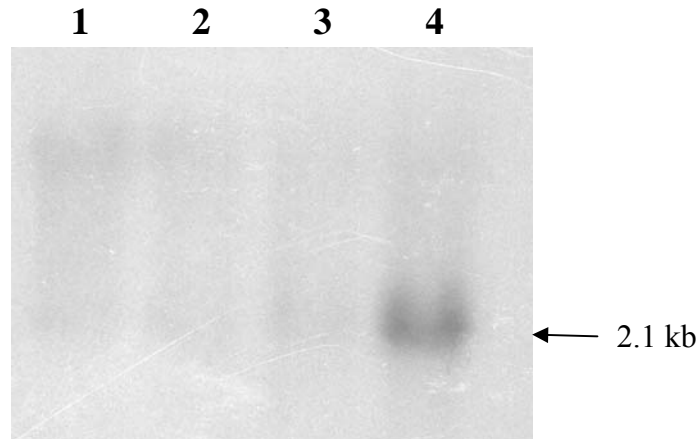


Figure 2. Northern blot analysis of RNA isolated from culture mouse mammary epithelial cells using a probe that detects *CRELD1-9b*. This probe does not cross-react with other *CRELD1* isoforms. Lane 1 contained the RNA from cells that were transfected with the MkbpA II vector with no insert (“empty vector”) cultured in the absence of dexamethasone. Lane 2 contained RNA from cells transfected with empty vector and cultured in the presence of dexamethasone. Lane 3 contained RNA from cells transfected with the Mkbp-CRELD1-9b construct and cultured in the absence of dexamethasone. Lane 4 contained RNA from cells transfected with the Mkbp-CRELD1-9b construct and cultured in the presence of dexamethasone. CRELD1-9b expression was detected only in lane 4. The 2.1 kb band is consistent with the expected size for CRELD1-9b mRNA. This demonstrates that the Mkbp-CRELD1-9b construct is expressed by mouse mammary epithelial cells under induction of the exogenous MMTV promoter when induced with dexamethasone. This experiment also demonstrated that there is tight control of expression of the endogenous construct.

- g. Appended subtask g. In order to determine if the mRNA produced from the expression construct is translated into protein, western blot analyses were performed using an antibody that detects CRELD1. Although the antibody is not specific for isoform 9b (it detects all CRELD1 isoforms), induction of protein product with dexamethasone will demonstrate that it comes from the inducible construct. Total protein from cultured mouse mammary epithelial cells transfected with the Mkbp-CRELD1-9b construct was run on SDS-PAGE and electrotransferred to membrane to produce the western blot, which was probed with CRELD-1 antibody. This demonstrated expression of protein under induction with dexamethasone, showing that the transcripts detected by northern blot are translated to protein product (Figure 3).

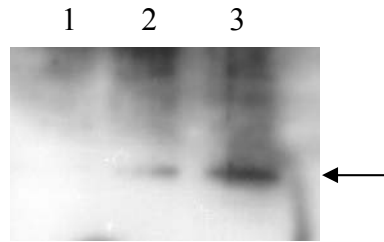


Figure 3. Western blot analysis of protein isolated from culture mouse mammary epithelial cells using an antibody that detects CRELD1. Lane 1 contained the protein from cells that were transfected with the MkbpA II vector with no insert ("empty vector) cultured in the absence of dexamethasone. Lane 2 contained protein from cells transfected with the Mkbp-CRELD1-9b construct and cultured in the absence of dexamethasone. Lane 3 contained protein from cells transfected with the Mkbp-CRELD1-9b construct and cultured in the presence of dexamethasone. Contrary to the data from northern blot analysis (Figure 2) there seems to be some leaky expression in the absence of dexamethasone (lane 2), or the antibody is detecting some exogenous CRELD1 expression. However, there does seem to be some significant induction of CRELD1-9b expression with the addition of dexamethasone (lane 3). This demonstrates that the Mkbp-CRELD1-9b construct expressed by mouse mammary epithelial cells produces a protein product.

*Task 2.* Creation of a transgenic mouse model for over-expression of CRELD1 isoform 9b in mammary tissue.

- a. High quality transgene DNA was produced and isolated as described. The DNA was analyzed by agarose gel electrophoresis and spectrophotometry (not shown).
- b. DNA was injected into embryos.
- c. Embryos were transferred into host mothers, pregnancies were completed and pups weaned.
- d. Pups were analyzed for presence of the transgene. The results showed that none of the animals carried the transgene. A second round of injections is being considered.

**KEY RESEARCH ACCOMPLISHMENTS:** Task 1 has been completed using an alternative approach that resulted in the successful construction of a mammalian expression vector system that is capable of transcribing exogenous CRELD1-9b under the control of dexamethasone induction. Expression of the construct was shown to produce protein product in cultured mammary cells. This has generated an inducible cell culture system that will be valuable for determining the role of CRELD1-9b. Although this was not a specified goal of this project, it is an important ancillary resource that resulted from the pursuit of the work here. This resource is being used to follow the consequences of CRELD1-9b expression on cell proliferation, adhesion, migration and survival.

**REPORTABLE OUTCOMES:** The preliminary results from this work were reported in an abstract and poster presentation at the Era of Hope 2005 Department of Defense Breast Cancer Program, Philadelphia PA, June, 2005.

**CONCLUSION:** A suitable expression system has been created that can be used to target expression of CRELD1-9b to mouse mammary epithelial cells in vivo. This also resulted in the generation of a cell culture system that will be of value in determining the effect of CRELD1-9b expression on cell

function. Data from experiments outside of the scope of this project suggest that an altered balance of CRELD1 and CRELD2 expression may play a role in cancer progression, or the aggressive behavior of cancer cells. We are using cell culture systems to better understand the interplay between CRELD1 and CRELD2 isoforms before continuing with animal experiments.

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Sternlicht MD, Lochter A, Sympton CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ, Werb Z. 1999. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 98:137-146.

APPENDICES: None

SUPPORTING DATA: Included in the BODY section.

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The Role of CRELD1 Isoform 9b in the Pathogenesis of Breast Cancer, Cheryl Maslen and Darcie Babcock, Era of Hope 2005 Department of Defense Breast Cancer Program, Philadelphia PA, June, 2005.

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